Mechanism of *in Vitro* Collagen Fibril Assembly

KINETIC AND MORPHOLOGICAL STUDIES*

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The kinetics of *in vitro* fibril assembly of Type I collagen preparations that contain different amounts of covalently cross-linked oligomers was studied with turbidimetry. Fibril formation showed a lag phase with no solution turbidity and a growth phase with a sigmoidal increase in the solution turbidity. The length of the lag phase was inversely related to both the total collagen concentration and the amount of covalently cross-linked oligomers in the solution. Double logarithmic plots of $t_{1/4}$, the amount of time it takes for ¼ of the collagen to assemble into fibrils, *versus* the total collagen concentration were linear but the slope decreased from −0.84 to −2.3 with decreasing amounts of covalently cross-linked oligomers in the samples. Electron microscopy showed the formation of unbanded microfibrils with diameters in the range of 3–15 nm early in the lag phase and larger diameter banded fibrils coexisting with the microfibrils near the end of the lag phase. Centrifugation of the solution at the lag phase prolonged the lag time, presumably by removal of microfibrils, but subsequent growth of the fibrils was unaffected. The results suggest a cooperative nucleation-growth mechanism for the *in vitro* assembly of collagen fibrils which is consistent with the results of an equilibrium study of the fibril assembly reaction we reported earlier (Na, G. C., Butz, L. J., Bailey, D. G., and Carroll, R. J. (1986) *Biochemistry* 25, 958–966).

The interstitial collagens are synthesized inside cells and secreted into extracellular space in the form of procollagen. There the propeptides are cleaved enzymatically and the collagen molecules polymerize into fibrils (Kivirikko and Myllyla, 1984). Currently, little is known regarding either the mechanism of formation of the collagen fibril from the monomer or the regulatory mechanism of the process. *In vitro*, solubilized collagen can be reconstituted into fibrils if incubated at 25 to 30 °C in a buffer with an ionic strength of 0.15 to 0.30 and a pH near neutral. The kinetics of the *in vitro* collagen fibril assembly consist of two phases, initiation and growth (Wood and Keech, 1960; Wood, 1960; Comper and Veis, 1977a, 1977b; Williams *et al.*, 1978; Gelman *et al.*, 1979a, 1979b). During the fibril initiation period, the solution does not display any turbidity, indicating that the large fibril structures have not yet formed. However, unbanded microfibrils of 3- to 5-nm diameter have been observed during the lag phase of fibril assembly (Veis *et al.*, 1979; Gelman *et al.*, 1979a). Growth of the fibril follows the initiation period and is characterized by a sigmoidal increase of the solution turbidity as well as the appearance of collagen fibrils with a 67-nm repeat band pattern.

There are few reported equilibrium studies of *in vitro* collagen fibril assembly. Wood and Keech (1960) observed that solubilized collagen polymerized entirely into fibrils under the assembly conditions. Nonetheless, a nucleation-growth mechanism was proposed on the basis of the sigmoidal characteristics of the reaction kinetics (Wood, 1960). More recently, Piez and co-workers confirmed that the fibril assembly reaction entails no critical concentration, *i.e.* the amount of fibrils formed, when plotted against the total collagen concentration, extrapolated to near the origin (Williams *et al.*, 1978; Gelman *et al.*, 1979a). Moreover, they carried out kinetic studies which showed a linear plot with a slope of −1 for the logarithm of the time required for half of the collagen to assemble into fibrils *versus* the logarithm of the total collagen concentration. On the basis of these observations, they suggested that the fibril assembly reaction proceeds through an accretion mechanism and does not involve the Oosawa helical cooperative assembly mechanism which has been shown in actin filament and microtubule formations (Oosawa and Kasai, 1962; Gaskin *et al.*, 1974). However, they indicated that their equilibrium data could not rule out the existence of a critical concentration of 7 μg/ml or less because of the limited sensitivity of the technique (Williams *et al.*, 1978).

If the fibril assembly does proceed through a cooperative mechanism with the initial formation of nucleation centers as suggested by Wood (1960), then there should be a critical concentration for the reaction, and its value should be inversely proportional to the polymer growth constant (Oosawa and Kasai, 1971; Timasheff, 1981). The reason that a critical concentration was not observed could be because the polymer growth was too strong. With this understanding, we added glycerol, which is known to inhibit the fibril assembly reaction, to the assembly buffer and demonstrated the presence of a critical concentration for the *in vitro* collagen fibril formation with its value inversely related to the glycerol concentration in the buffer (Na *et al.*, 1986). Therefore, we proposed a helical cooperative mechanism for the fibril assembly reaction (Na *et al.*, 1986), adopting as an intermediate the five-stranded microfibrils originally conceived by Smith (1968).

While the results of our equilibrium study support the helical cooperative mechanism for collagen fibril assembly, the kinetic evidence reported by Piez and co-workers remained contradictory (Williams *et al.*, 1978; Gelman *et al.*, 1979a, 1979b). It is true that a slope of −1 in the abovementioned double logarithmic plot is inconsistent with a cooperative nucleation-growth association mechanism. However, the kinetic equation was derived under the assumption

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that the polymerization reaction starts exclusively from the monomer (Oosawa and Kasai, 1971; Purich and Kristofferson, 1984). Because of the possible presence of covalently cross-linked oligomers[1] in the collagen solutions used in the earlier kinetic studies (Chandrakasan *et al.*, 1976; Williams *et al.*, 1978; Gelman *et al.*, 1979a), their interpretation may not be valid. In order to further substantiate the cooperative spontaneous nucleation-growth mechanism of collagen fibril assembly and to determine the stoichiometry of the nucleation center, calf skin collagens that contain different amounts of oligomer were purified and the kinetics of their fibril formation was examined. The results are reported here.

MATERIALS AND METHODS

UV-grade guanidine hydrochloride (GdnHCl[2]) was purchased from United States Biochemical Corp.[3] and solutions were filtered through a sintered glass funnel before use. Spectranalyzed glycerol was from Fisher. *p*-Chloromercuribenzoate and phenylmethanesulfonyl fluoride were from Sigma. All other chemicals used were reagent-grade.

Preparation and Concentration Determination of Calf Skin Collagen—Type I collagen was extracted from fresh calf skin obtained from a local slaughter house. The purification procedure was essentially that described in a previous publication (Na *et al.*, 1986), except that 1 M glycerol was added to the extraction buffer to stabilize the collagen against denaturation and to facilitate the solubilization of the protein (Na, 1986). In several preparations, three protease inhibitors (0.02 M sodium EDTA, 1 mM *p*-chloromercuribenzoate, and 10 μM phenylmethanesulfonyl fluoride) were also added to the extraction buffer to prevent endogenous enzymatic digestion of the nonhelical telopeptides (Miller and Rhodes, 1982). The collagen extracted in the absence of the protease inhibitors is called "noninhibitor collagen" whereas the one extracted in the presence of the protease inhibitors is called "inhibitor collagen" hereafter.

The protein concentration was determined by diluting a small aliquot of the sample with 3 volumes of 8 M GdnHCl and an appropriate volume of 6 M GdnHCl and measuring the solution absorbance at 218 nm. An absorption coefficient of 9.43 $ml \cdot mg^{-1} \cdot cm^{-1}$ was used (Na *et al.*, 1986).

The tyrosine content of the protein was determined by UV spectroscopy (Na *et al.*, 1986). 0.3 ml of the stock collagen was diluted with 0.6 ml of 9 M GdnHCl, 0.03 M NaP_i, pH 6.5. The solution showed an absorption peak at 276 nm. Since collagen does not contain any tryptophan residues, a molar absorption coefficient of 1500 $liters \cdot mol^{-1} \cdot cm^{-1}$ at 276 nm was used for the tyrosine residue (Edelhoch, 1967). A collagen molecular weight of 285,000 based on the amino acid composition of the protein was used in the calculation (Miller, 1984).

Preparation of Monomeric and Oligomeric Collagens—Calf skin collagen isolated as described above is referred to as "crude collagen." SDS-polyacrylamide gel electrophoresis showed that this preparation contained components with molecular weights greater than that of the γ band (M_r = 285,000), reflecting the presence of covalently cross-linked oligomers. To remove the collagen oligomers, the protein was dissolved in PS buffer (0.03 M NaP_i and 0.1 M NaCl, pH 7.0) containing either 0.6 M (noninhibitor collagen) or 0.8 M (inhibitor collagen) glycerol and incubated at 30 °C for 24–72 h. The collagen fibrils formed were separated by centrifugation at 12,000 × *g* for 10 min. The pellets were dispersed in 0.1 M HOAc and dialyzed exhaustively against 1 mM HOAc. This collagen will be referred to as "oligomeric collagen." The protein in the supernatant was then precipitated by adding solid NaCl to 3 molal and collected by the same centrifugation. These pellets, too, were dispersed in 0.1 M HOAc, dialyzed overnight against 1 mM HOAc, and stored under liquid nitrogen. The latter collagen will be referred to as "monomeric collagen."

SDS-Polyacrylamide Gel Electrophoresis—The oligomer content of the collagen was determined by electrophoresis using 4% SDS-polyacrylamide disc gels prepared by following the method of Murray *et al.* (1982). 80 to 90 μg of collagen was loaded onto each gel. The gel was initially run for a shorter time to confirm that the two α bands were the lowest molecular weight components. A longer running time of 10.5 h was subsequently adopted in order to obtain better resolutions of the high molecular weight bands. The gels were stained with Coomassie Brilliant Blue R-250. The percent distribution of the collagen among different bands was determined by scanning the gel at 580 nm using a Shimadzu Model SC-930 densitometer.

Kinetics of in Vitro Collagen Fibril Assembly—The kinetics of the *in vitro* self-assembly of solubilized collagen into fibrils was measured in PS buffer. A concentrated collagen solution at approximately 2 to 3 mg/ml in PS buffer was prepared by adding to PS buffer a small aliquot of the stock collagen, usually around 10 mg/ml in 1 mM acetic acid, and an equal volume of a double strength PS buffer. The mixture was dispersed through gentle shaking at 4 °C. A Microman pipette (Rainin Instrument Co. Inc., Woburn, MA) with a piston-type disposable tip was used to measure and deliver the viscous collagen stock solution. Unless otherwise specified, the concentrated collagen solution was passed through a Millex-GV 0.22-μm filter (Millipore) before its concentration was measured. The formation of collagen fibrils was monitored by measuring the solution turbidity at 350 nm using a Perkin-Elmer Lambda-7 UV-visible spectrophotometer. Both the cuvettes and the cell holder were jacketed and connected in series with a circulating water bath. An automatic cell changer allowed simultaneous measurement of up to six samples. Two methods were used to initiate the fibril assembly. In the first method, 800 to 900 μl of PS buffer was added to each cuvette and was equilibrated at 30 °C. An aliquot of the concentrated collagen solution in PS buffer at 10 °C was then added to each cuvette to bring the total volume to 1 ml. The solution was mixed by turning the cuvette upside down three times and the turbidity measurement was then started. With this method, the solution warmed up to 30 °C within 10 s. It is thus suitable for samples such as the oligomeric collagen with rapid rates of fibril assembly. In the second method, the cuvettes were kept at room temperature (20–25 °C) whereas the collagen solutions were maintained at 10 °C. The solutions were degassed under vacuum for 2 min and added to the cuvettes. This was followed immediately by opening the valve of the water bath and starting the turbidity measurement. The temperature of the sample reached 30 °C within 2 min. Care was taken to ensure that no air bubble was created during the transfer of the solution to the cuvette. This method was used with the monomeric collagens that have slow rates of fibril assembly. With the monomeric collagen, the extra care of avoiding vigorous mixing and eliminating air bubbles is essential for obtaining consistent results. Since the rates of fibril formation of these samples are slow, the 2-min warm-up time can be ignored.

The validity of the solution turbidity as a quantitative measure of the weight concentration of collagen fibrils was verified experimentally by comparing the turbidity results with those of direct measurement of fibril concentration (Na *et al.*, 1986). In the latter method, the amount of fibrils formed was determined by centrifuging the fibril solution in a 1.5-ml microcentrifuge tube at 12,000 × *g* for 2 min using a Sorvall SH-MT rotor. The protein concentrations of both the solution prior to the assembly and the supernatant after the centrifugation were measured and the difference between them was taken as the amount of fibrils formed. Both the kinetics and the equilibrium of the fibril assembly reaction were examined in this manner. Fig. 1 shows the kinetics of fibril assembly measured by both turbidimetry (*solid curve*) and direct concentration determinations (*circles*). It is evident that these two sets of data differed only at the lag phase where the direct concentration measurements consistently showed the formation of small amounts of sedimentable aggregates before the appearance of the solution turbidity. The growth and plateau stages of the fibril assembly measured by the two methods were identical.

Fig. 2 shows the equilibrium of the fibril assembly measured by these two techniques. The *inset* of this figure indicates that, at the plateau of the fibril assembly carried out in PS buffer, collagen assembled essentially entirely into fibrils, in agreement with what we reported earlier (Na *et al.*, 1986). The main figure shows plots of the solution turbidity (absorbance), measured at 350 nm at the plateau of the fibril assembly, against collagen concentration. For the crude and monomeric collagens, the solution turbidity increased linearly with the total collagen concentration below 0.2 mg/ml. Above about 0.2 mg/ml, the data deviated from the straight line. In the linear

[1] In the present context, the terms "monomer" and "oligomer" refer to the M_r 285,000 triple-helical collagen molecule and the covalently cross-linked species with molecular weights greater than 285,000, respectively.

[2] The abbreviations used are: GdnHCl, guanidine hydrochloride; Ac, acetyl; SDS, sodium dodecyl sulfate.

[3] Reference of company or product names does not constitute endorsement by the United States Department of Agriculture over others of similar nature.

TABLE I

Oligomer contents and fibril assembly kinetics of calf skin collagens

Sample	Collagen Type[b]		Distribution[a] α	β	γ	HMW[c]	Filtration[d]	$\frac{d \ln t_{1/4}}{d \ln c_0}$	$\frac{d \ln t_{lag}}{d \ln c_0}$
		mg/ml[e]		%					
Noninhibitor collagen									
1	C		50.1	31.5	12.0	6.4	−	−1.06 ± 0.03	−1.04 ± 0.02
							+	−1.18 ± 0.04	−1.05 ± 0.07
2	O	1.30	37.7	35.6	11.1	15.6	−	−0.88 ± 0.06	−0.81 ± 0.07
							+	−0.84 ± 0.02	−0.83 ± 0.01
3	M	0.70	54.6	34.2	9.9	1.3	+	−1.32 ± 0.02	−1.24 ± 0.03
4	M	0.90	59.3	31.0	9.2	0.4	+	−2.00 ± 0.13	−1.73 ± 0.12
5	M	1.10	60.5	31.0	8.4	0.2	+	−2.26 ± 0.05	−2.14 ± 0.17
6	M	1.30	61.3	31.4	7.3	0.0	+	—[f]	—[f]
Inhibitor collagen									
7	C		45.5	32.5	10.2	11.8	−	−1.03 ± 0.03	−0.99 ± 0.09
							+	−1.20 ± 0.05	−1.20 ± 0.06
8	O	2.19	37.1	33.5	12.3	17.2	−	−1.10 ± 0.06	−1.24 ± 0.20
							+	−1.10 ± 0.05	−1.29 ± 0.12
9	M	0.96	53.0	36.3	7.5	3.2	+	−1.23 ± 0.07	−1.21 ± 0.06
10	M	1.58	54.1	33.9	9.6	2.4	+	−1.39 ± 0.08	−1.34 ± 0.11
11	M	2.19	61.6	31.7	5.1	1.6	+	−2.29 ± 0.22	−2.29 ± 0.34

[a] These are the percent distribution of collagen in bands in SDS-gel electrophoreses as measured by a gel scanner.

[b] The letters C, O, and M stand for crude, oligomeric, and monomeric collagen, respectively.

[c] The high molecular weight (HMW) consists of all components with molecular weight greater than that of the γ band.

[d] Prior to the kinetic measurement of fibril assembly, the concentrated collagen solutions were filtered with Millipore Millex-GV (0.22 μm) filters except for samples 2 and 8, which were filtered with Millex-PF (0.80 μm) filters.

[e] These are the collagen concentrations used in the glycerol-PS buffer to separate the oligomers from the monomeric collagen.

[f] These results were unattainable due to poor experimental accuracy (see "Discussion").

phoresis patterns of the noninhibitor collagens containing different amounts of oligomer. The crude collagen (*Lane 1*) displayed two α bands (α1 and α2, M_r = 95,000), two β bands (β_{11} and β_{22}, M_r = 190,000), two γ bands (γ_1 and γ_2, M_r = 285,000), and at least three high molecular weight bands (*HMW*, M_r > 285,000). The high molecular weight bands collectively comprised 6% of the total protein (Table I). *Lanes 2* to *6* show monomeric collagens that were purified through the fibril assembly procedure. They differed in the collagen concentration used in the fibril assembly which ranged from 0.3 to 1.3 mg/ml. These gel patterns indicate that the high molecular weight components can be removed more thoroughly by using a higher collagen concentration in the glycerol-PS fibril assembly buffer. As indicated by the percent distribution of collagen among the different bands listed in Table I, by increasing the collagen concentration used in the fibril assembly, the relative percent of the high molecular weight bands and the γ_2 band diminished gradually, whereas those of the α1, α2, β_{11}, β_{22}, and γ_1 bands increased slightly in balance. By using a collagen concentration of 1.3 mg/ml in the fibril assembly, the monomeric collagen subsequently obtained exhibited no trace of either the γ_2 band or the high molecular weight bands (Fig. 3, *Lane 6*).

The gel patterns of the inhibitor collagens (not shown) were qualitatively identical to those of the corresponding noninhibitor collagens. The relative distributions of the individual bands of the inhibitor collagens are listed at the bottom half of Table I.

Kinetics of Fibril Assembly of the Noninhibitor Collagen—As shown in Fig. 4, the kinetics of the *in vitro* collagen fibril assembly can be divided into two phases. After the solution is heated to 30 °C, there is usually a lag phase with no change in solution turbidity. This is followed by a growth phase with a sigmoidal increase of the solution turbidity. The length of

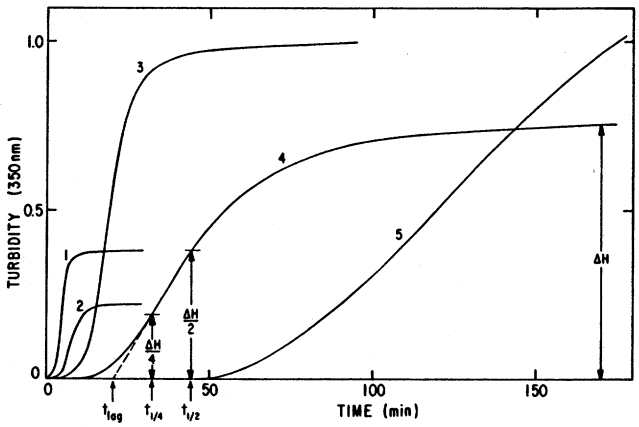

FIG. 4. **Kinetics of fibril assembly from noninhibitor collagens.** The reaction was monitored by measuring the solution turbidity at 350 nm. The length of the lag phase was obtained by linearly extrapolating the growth phase to the base line. Also shown are the definitions of $t_{1/4}$ and $t_{1/2}$. The curves numbered *1* to *5* correspond to oligomeric collagen at 0.188 and 0.106 mg/ml, crude collagen at 0.183 and 0.107 mg/ml, and monomeric collagen at 0.204 mg/ml, respectively.

the lag phase was measured by extrapolating the linear region of the growth phase to the base line. As shown by *curves 1–4* of Fig. 4, for the crude and oligomeric collagens, the lag time increased with decreasing total collagen concentration. This was also true for the monomeric collagen. Comparing at the same concentration the oligomeric, crude, and monomeric collagens, the lag phase lengthens in the above order, apparently reflecting the decreasing amount of oligomers in the sample. In fact, at concentrations above 0.3 mg/ml the solution turbidity of the oligomeric collagen seemed to emerge instantaneously after heating and did not display a measura-

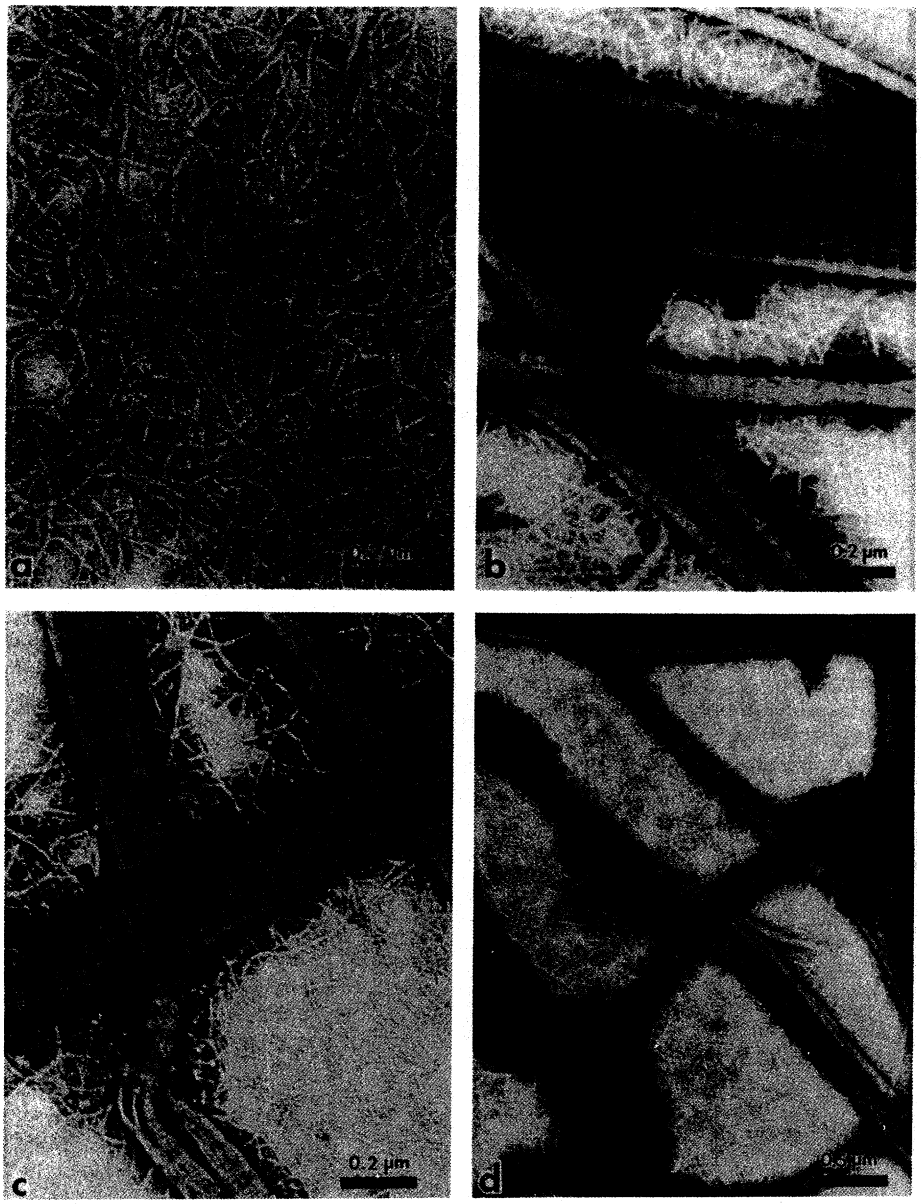

FIG. 7. **Electron micrographs of collagen aggregates formed at various stages of fibril assembly.** The sample was inhibitor collagen at 0.12 mg/ml. It was applied to the grid after 10 min (*a*) and 20 min (*b–d*) of incubation at 30 °C. In *a*, microfibrils of different diameters ranging from 3 to 15 nm were observed. *b–d* show the coexistence of unbanded microfibrils with banded fibrils toward the end of the lag phase. In *b*, loosely packed fibrils with faint band patterns were evident, whereas in *c* and *d* splitting of a single fibril into microfibrils was observed.

DISCUSSION

As stated in the Introduction, the general features of the kinetics of *in vitro* fibril assembly of type I collagen have been known for decades. The assembly of collagen fibrils usually starts with a lag period showing no solution turbidity, the length being inversely related to the collagen concentration. This is followed by a growth phase with a sigmoidal increase of the solution turbidity and the appearance of banded fibrils (Wood, 1960; Wood and Keech, 1960; Comper and Veis, 1977a, 1977b; Williams *et al.*, 1978; Gelman *et al.*, 1979a). Such assembly kinetics are characteristic of a nucleation-growth mechanism which has been shown to be true in the *in vitro* assemblies of actin filaments and microtubules and it has led to the initial proposal of the nucleation-growth mechanism for collagen fibril assembly (Wood, 1960). However, more recent analyses of fibril assembly kinetics by a double logarithmic plot defined above have shown a straight line with a slope of −1 (Williams *et al.*, 1978; Gelman *et al.*, 1979a, 1979b). This finding apparently refutes the cooperative nucleation-growth mechanism and lends support to an accretion mechanism for collagen fibril assembly.

The present study addresses mainly the effect on the kinetics of fibril assembly of the covalently cross-linked oligomers found in most collagen preparations and its implication on the mechanism of fibril assembly derived from the kinetic studies. In addition to the sigmoidal shape of the kinetics of fibril assembly, the observation that the more the oligomer in the solution the faster the rate of fibril assembly suggests a cooperative nucleation-growth mechanism for the reaction. For this mechanism, the monomeric collagen is expected to self-associate and form nucleation centers more slowly than the oligomeric collagen. The latter either already contains the nucleation centers or can form the nucleation centers much faster and, consequently, their lag phase should be much shorter. Since the kinetics of the *in vitro* collagen fibril assembly displayed all the signs of a cooperative nucleation-growth polymerization, the data were analyzed accordingly. A cooperative self-association involving spontaneous nucleation and growth from the monomer can be expressed as (Oosawa and Kasai, 1971; Timasheff, 1981; Na *et al.*, 1986):

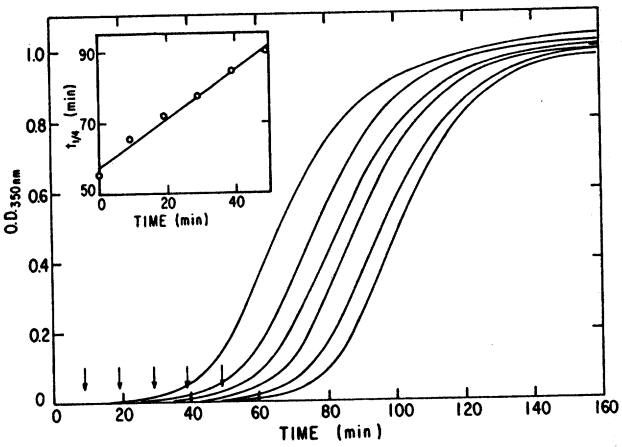

FIG. 8. **Effects of centrifugation at lag phase on the kinetics of fibril assembly.** The crude inhibitor collagen at 0.16 mg/ml in 0.03 M NaP_i, 0.15 M NaCl, pH 7.0, buffer was used. The control was added directly to a jacketed cuvette at 30 °C and fibril assembly is represented by the curve farthest to the *left*. *Curves 2–6*, counting from the *left*, depict fibril assembly of samples which were incubated in 1.5-ml Microfuge tubes at 30 °C for increasing amounts of time indicated by the *arrows* and centrifuged at 12,000 × *g* for 2 min, and each supernatant was then added to a jacketed cuvette at 30 °C to monitor fibril assembly with turbidimetry. The *inset* shows a plot of $t_{1/4}$ *versus* the sample's incubation time prior to the centrifugation. The *straight line* is the linear least squares fit of the data which has a slope of 0.68.

$$\begin{array}{lll} A + A \rightleftharpoons A_2 & K_L \\ A_2 + A \rightleftharpoons A_3 & K_L \\ \vdots \quad \vdots \quad \vdots & \vdots \\ A_{n-1} + A \rightleftharpoons A_n & K_L \end{array} \qquad (1)$$

where A_i represents the i-mer and $[A_i]$ is its molar concentration. K_L is the equilibrium constant for each step of the self-association. As the stoichiometry of the linear polymer reaches a certain value n, it closes itself into a loop-structured nucleation center:

$$A_{n\,(\text{linear})} \rightleftharpoons A_{n\,(\text{circular})} \qquad K_C \qquad (2)$$

where K_C is the equilibrium constant between the linear n-mer and the circular n-mer. After the formation of the nucleation center, the elongation stage sets in. The elongation or growth of the polymer is similar to the initiation stage except each step of the association has a more negative free energy change. Its reaction equilibria and kinetics can be expressed as:

$$\begin{array}{lll} A_{n\,(\text{circular})} + A \underset{k_-}{\overset{k_+}{\rightleftharpoons}} A_{n+1} & K_H \\ A_{n+1} + A \rightleftharpoons A_{n+2} & K_H \\ \vdots \quad \vdots \quad \vdots & \vdots \\ A_{n+j-1} + A \rightleftharpoons A_{n+j} & K_H \end{array} \qquad (3)$$

where K_H is the equilibrium constant for the incorporation of one monomer to the growing end of the polymer. k_+ and k_- are the rate constants for the association and dissociation of a monomer from the growing end of the polymer, respectively. If the polymerization is cooperative, then the amount of intermediate oligomers between the monomer and n-mer will be insignificant and the rate of formation of the nucleation center from the monomer can be expressed as:

$$nA \underset{k_-^*}{\overset{k_+^*}{\rightleftharpoons}} A_n \qquad (4)$$

where k_+^* and k_-^* are the apparent association and dissociation rate constants. It has been shown that the kinetics of a cooperative polymerization proceeding through the above nucleation-growth mechanism can be described by (Oosawa and Kasai, 1962; Oosawa and Asakura, 1975):

$$\ln \frac{1 + [1 - (c_1/c_0)^n]^{1/2}}{1 - [1 - (c_1/c_0)^n]^{1/2}} = 2n^{1/2}(k_+k_+^*)^{1/2}c_0^{n/2}t \qquad (5)$$

where t is time, c_0 is the total weight concentration of the protein, c_1 is the weight concentration of the monomer, and n is the stoichiometry of the nucleation center. By adopting a constant ratio of c_1/c_0, Equation 5 can be reduced to:

$$\ln t = \frac{-n}{2} \ln c_0 + \text{constant} \qquad (6)$$

which indicates that for such a system if one plots ln t, t being the time it takes for a given percentage of the monomer to polymerize, *versus* ln c_0, the data should be linear with a slope of $-n/2$, n being the stoichiometry of the nucleation center. It should be noted, however, that this rather simple kinetic equation was derived under three important assumptions. First, it was assumed that the rate of dissociation of the monomer from the polymer is very slow and can be ignored. Second, it was assumed that during polymer growth only the monomer becomes associated with the polymer. Polymers do not associate with one another and they do not dissociate into smaller polymers. In other words, the nucleation center can be formed only from linear polymerization of the monomers and not from breaking down of the existing polymers. Third, it was assumed that the monomer is the only species in the solution at the beginning of the polymerization. We shall consider these three assumptions in turn.

The first condition can be met by taking a ratio of c_1/c_0 near 1, i.e. examining the initial rate of fibril assembly where the concentration of the polymer is low and its dissociation can be ignored. In the data analysis shown above, $t_{1/4}$ was used instead of $t_{1/2}$ as in earlier studies to better satisfy this condition. In fact, one can extrapolate the linear portion of fibril growth to the base line to obtain the length of the lag phase which represents the amount of time it takes for the formation of an infinitesimal amount of fibrils. As shown in the last column of Table I, double logarithmic plots of t_{lag} *versus* the collagen concentration gave slopes very similar to those of $t_{1/4}$. Such an analysis should alleviate the problems of polymer dissociation and the nonlinearity of the turbidity at high fibril concentrations shown in Fig. 2.

The second assumption mentioned above can also be assumed to be reasonably satisfied in the case of collagen fibril formation. If the fibril assembly proceeds through microfibrils as an intermediate as suggested by the results of Fig. 7*a*, it is unlikely that a significant amount of polymer can break down into smaller polymers. Such a polymer fragmentation would require a rather high activation energy. It is equally unlikely that there is a significant amount of end-to-end longitudinal association among the microfibrils or fibrils because these large asymmetrical structures should have slow translational and rotational movements and could not provide frequent enough mutual collisions with proper orientation of the molecules to result in polymer association.

With respect to the third assumption, the absence of oligomers from the collagen solution is uncertain. Depending on

the age of the animal and the method of extraction, type I collagen obtained from skin or tendon usually contains a few percent of oligomers that are covalently cross-linked. The presence of covalent intercollagen cross-links is usually reflected in the appearance of component(s) with molecular weights greater than 285,000 under denaturing conditions such as in SDS-polyacrylamide gel electrophoresis. If polymer formation requires the initial formation of nucleation centers, it is reasonable to expect the oligomers in the solution to accelerate the rate of polymer formation. This effect could be particularly pronounced at low protein concentrations where spontaneous nucleation is slow but to a lesser degree at high protein concentrations where spontaneous nucleation is fast. As a result, the presence of the oligomers could significantly change the slope of the double logarithmic plot. Indeed, in Figs. 5 and 6, the slopes of the double logarithmic plots of the kinetics of fibril formation from crude collagens became more negative if the protein was passed through a 0.22-μm filter before being assembled. This observation suggested that there were aggregates in the solution affecting the kinetics of the assembly reaction which were retained by the 0.22-μm filter. The 0.8-μm filter seemed to have no measurable effect on the kinetics of fibril assembly, suggesting that the aggregates are relatively small in size. While these are qualitative observations, subsequent studies using collagen preparations that contained different amounts of oligomer confirmed that the slope is indeed inversely related to the oligomer content of the solution.

According to Equation 6, a slope more negative than -1 in the double logarithmic plot indicates that the fibril assembly reaction proceeds through a cooperative mechanism. In fact, a slope of -2.3 was obtained with both a noninhibitor and an inhibitor collagen sample that contained, respectively, 0.2 and 1.6% high molecular weight components as determined by SDS-polyacrylamide gel electrophoresis, suggesting that the nucleation center for the cooperative self-association could be a pentamer. It is interesting to note that this stoichiometry agrees with the Smith five-stranded microfibril model in which parallel collagen monomers are staggered by 1 D[5] and packed in a helical manner (Smith, 1968). It should be emphasized, however, that, given the uncertainty of the slopes shown in Table I and the fact that a definitive slope could not be obtained for a monomeric collagen that contains absolutely no high molecular weight components, our results by no means rule out other association models that employ nucleation centers of slightly higher stoichiometries.

In juxtaposition with the kinetic analyses we examined by electron microscopy the morphologies of the collagen aggregates formed at various phases of the assembly reaction. The electron micrographs shown in Fig. 7*a* confirmed the results reported earlier by Gelman *et al.* (1979a) indicating that collagen forms microfibrils during the lag phase of fibril assembly. It appears, as originally proposed by Gelman *et al.* (1979a), that the small diameters and the low concentration of these microfibrils render them undetectable by turbidity measurement. Unlike the fibrils, the microfibrils shown in Fig. 7*a* are unbanded. One explanation for the lack of banding is as follows. According to the model of D-staggered packing of collagen molecules within fibrils, the band pattern of a negatively stained collagen fibril arises from a heavier deposit of stain molecules at the 0.6 D gap between two coaxial collagen molecules (Hodge and Petruska, 1963; Petruska and Hodge, 1964; Hodge, 1967). One expects, as is true in large diameter fibrils, that in order for the extra stain to remain in the gap, this area must be enclosed by neighboring collagen molecules. Unlike the fibrils, a small diameter microfibril, assuming that it has the same D-staggered packing of collagen molecules as proposed by Smith (1968), will have these void areas exposed to the solvent and consequently may not be able to retain the extra stain to show the band pattern. It is interesting to note that when the fibrils first emerged at the end of the lag phase some of the band patterns appeared faint (Fig. 7*b*) suggesting that the microfibrils were still loosely packed within the fibrils.

The results of Fig. 8 showed that the removal of the microfibrils through centrifugation prolonged the lag phase but did not affect the kinetics of fibril growth. This confirmed that the formation of microfibrils indeed takes place in the lag phase and suggested that the microfibrils serve as an intermediate for the subsequent fibril growth. The *inset* of Fig. 8 shows a plot of $t_{1/4}$ *versus* the incubation time prior to the centrifugation of the sample. The data were linear and showed a slope of 0.68, suggesting that only a fraction of the potential fibril growth sites, perhaps only those with higher molecular weights, were removed by the relatively slow and short centrifugation.

The formation of microfibrils during the *in vitro* fibril assembly prompted re-examination of the meaning of the kinetic data analysis. As mentioned earlier, the solution turbidity measured only the concentration of the fibrils and not the microfibrils. On the other hand, the agreement between the kinetics of fibril assembly measured by turbidimetry and by centrifugation (Fig. 1) suggested that the amount of microfibrils present is small (<5%) at all phases of fibril assembly. This is supported by the results of Fig. 8 which show only a slight decrease of the solution turbidity at the plateau of the assembly as a result of centrifugation at the lag phase. The limited quantity of microfibrils during fibril assembly suggested that the rate-limiting step of the reaction probably lies at the initial formation of the microfibrils from monomers and not the subsequent formation of fibrils from microfibrils. Consequently, the stoichiometry derived from the double logarithmic plot of the kinetic data should reflect that of the nucleation center for the formation of the microfibrils.

In this study collagen was extracted both in the presence and absence of protease inhibitors. The protein from both preparations showed the same tyrosine content of 12 ± 1 residues/collagen, suggesting that the nonhelical telopeptides remained intact. This is consistent with the observation of Chandrakasan *et al.* (1976) that the absence of the protease inhibitors would not reduce the tyrosine content of the collagen if the initial solubilization step was carried out at 4 °C for 24 h or less. Gel electrophoreses of the inhibitor and noninhibitor collagens showed the same bands with similar weight distributions. However, the two collagen preparations did display different critical concentrations of fibril assembly in 0.8 M glycerol-PS buffer, 1.2 mg/ml for the noninhibitor collagen but 0.4 mg/ml for the inhibitor collagen. This suggests that fibril growth is stronger for inhibitor collagen than noninhibitor collagen by approximately 0.7 kcal/mol (Na *et al.*, 1986). The kinetic study showed the rate of fibril assembly of the inhibitor collagen to be faster than that of the noninhibitor collagen, which is consistent with the equilibrium results. The inhibitor collagen showed a higher oligomer content which may also have contributed to the faster rate of fibril assembly. Alternatively, it is also possible that the noninhibitor collagen had a few amino acid residues deleted from its N- and/or C-terminal telopeptides by endogeneous proteases during extraction. This may not result in a change of the tyrosine content of the protein, since in α1 chains the

[5] The unit D is equal to 67 nm, the combined length of the dark band and light band of the collagen fibril.

first tyrosine residue is located at the fourth position from the amino end and the third position from the carboxyl end (Miller, 1984). Such a deletion could weaken and slow fibril assembly (Helseth and Veis, 1981; Capaldi and Chapman, 1982, 1984).

With decreasing amounts of oligomer in solution, a gradual deterioration of the reproducibility of the fibril assembly kinetics was noted. In Table I, sample 6 showed no measurable amount of high molecular weight component. However, the rate of fibril assembly of this collagen in PS buffer was very slow and unreproducible. In particular, the duration of the lag phase varied substantially from experiment to experiment. This phenomenon is akin to many crystallization reactions in that environmental conditions such as vibration (from the automatic cell changer), the condition of the surface of the vessel, and a minute amount of impurity can greatly influence the rate of the reaction. It seems to reflect an extreme difficulty in the formation of the nucleation center but a much more favorable growth of the polymer once the nucleation center has taken its shape. Again, this is consistent with and supportive of a nucleation-growth mechanism for fibril assembly.

The preferential polymerization of collagen oligomers into fibrils in glycerol buffer could be explained in terms of either an equilibrium or kinetic effect. In an earlier study, it was noted that glycerol is preferentially attracted to the protein and that surface contact between collagen and glycerol solution is energetically favorable (Na, 1986). The oligomers have smaller surface areas per unit weight than the monomer and, therefore, should be less stabilized by the presence of glycerol in the solution than the monomer. Consequently, from the chemical equilibrium point of view, in glycerol solution the oligomers should have stronger propensities to form fibrils than the monomer. The presence of glycerol can also greatly reduce the rate of fibril assembly and perhaps the rate of the nucleation process. Since the oligomers, particularly those with higher molecular weights, can serve as nucleation centers for polymerization, from the kinetic point of view they should also become preferentially polymerized. Indeed, our study showed that simply carrying out the fibril assembly in PS buffer, stopping the assembly reaction before it reaches a plateau, and then pelleting the fibrils will not result in a measurable enrichment of the collagen monomer in the supernatant. Therefore, it is the glycerol in the solution that has led to the preferential polymerization of the oligomer. The presence of glycerol introduces another advantage in that it generates a critical concentration for the fibril assembly reaction (Na *et al.*, 1986). As a result, after long incubation at 30 °C, one is assured of obtaining a solution of unpolymerized collagen at the critical concentration of fibril assembly. As expected, the higher the initial collagen concentration, the more thoroughly the oligomers were removed. This method can serve as an alternative for obtaining rather pure monomeric Type I collagen when it is either impractical or undesirable to feed an animal with a lathyritic agent to suppress the formation of intercollagen cross-links in order to obtain monomeric collagen.

It has been reported that NaCl can salt out preferentially the oligomeric collagen (Chandrakasan *et al.*, 1976). A collagen at 3 to 4% salt cutoff was used by Piez and co-workers in their kinetic study of fibril assembly (Williams *et al.*, 1978; Gelman *et al.*, 1979a, 1979b). According to their earlier column gel filtration studies, it should contain anywhere from 2 to 5% of high molecular weight components (Chandrakasan *et al.*, 1976). However, their more recent reports stated that, by using tissue from younger animals and by adding NaCl solution rather than crystal, the resulting collagen contained only a trace amount of high molecular weight components (Williams *et al.*, 1978; Gelman *et al.*, 1979a, 1979b). The crude collagen used in the present study precipitated almost entirely between 3.0 and 3.5% NaCl, suggesting that it should be comparable in its oligomer content to the rat tendon collagen used by Piez and co-workers. This is supported by the agreement of the slope of the double logarithmic plot of the fibril assembly kinetics obtained from our crude collagen with their results. The complete precipitation of the crude collagen used in the present study at 3.5% NaCl also indicated that salt precipitation is unable to remove the last 1 or 2% of oligomer from the sample (Chandrakasan *et al.*, 1976). Yet, this small amount of oligomers evidently can exert a significant effect on the kinetics of fibril assembly.

It is interesting to note that fibrils formed from the oligomeric collagen displayed a substantially lower turbidity than those formed from crude and monomeric collagens. McPherson *et al.* (1985) reported that the solution turbidity of fibrils assembled from pepsin-solubilized bovine collagen was inversely related to the rate of fibril formation. Their morphological study indicated that the lower solution turbidity can be attributed to reduced fibril diameter. In the present study, the oligomeric collagen showed a faster rate of fibril initiation than the crude and monomeric collagen. It is thus likely that a greater number of nucleation centers were formed from oligomeric collagen during the initiation period. Consequently, oligomeric collagen could have formed shorter fibrils that scatter less light. This possibility needs to be investigated further.

In summary, the kinetics of the *in vitro* assembly of monomeric calf skin collagen into fibrils can be well described by a classic cooperative nucleation-growth mechanism. Electron microscopic studies showed the formation of microfibrils at an early stage, *i.e.* during the turbidity lag of fibril assembly. Double logarithmic plots of the kinetic data suggested that the nucleation center for the fibril assembly could be pentamers, although oligomers of similar stoichiometries were not ruled out. It appeared that the banded fibrils were formed through lateral parallel packing of the microfibrils. This mechanism of fibril assembly, derived from the kinetic data, is fully consistent with the conclusions we reached earlier from equilibrium observations (Na *et al.*, 1986).

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